The University of Chicago

IMMUNOLOGIC STUDIES IN POLIOMYELITIS

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1935

By

JAMES ALEXANDER HARRISON

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INTRODUCTION

One of the most pressing needs of the poliomyelitis worker today is a simple and accurate method of testing for immunity in recovered human beings and monkeys. Since Levaditi and Landsteiner's demonstration of the inactivating effect of the serum of convalescent persons on the virus of poliomyelitis, a considerable amount of work has been done with this reaction. The test is not, however, a suitable diagnostic method for several reasons, three of which are: it is inaccurate in so far as individual tests are concerned, particularly if the serum antibodies are in relatively low dilution; it has been objected to on the grounds that it has not been proved to be a specific test for immunity; and, it is at best highly impractical in application except on a small scale.

Practically any type of test tube or skin reaction, if specific, which could be applied in large numbers would make possible a great increase in the amount and dependability of work that could be done, with no increase in material outlay. The advantages of this are so numerous and obvious that they need no discussion here. A number of investigations have been conducted in efforts to produce such a test, and with but few exceptions the results have been negative.

The solution to the problem seems to lie in re-searching the fields already studied, perhaps from new and different angles of approach, in analysing the serum-neutralization reaction in an effort to determine its dependability, or in searching for newer

methods of diagnosis. While it has been impossible to carry out such a comprehensive study in an exhaustive fashion with limited means, each phase of the problem has been considered to some extent. This was done in order to make the best use of materials which had been prepared and tested in animals.

The present researches have been concerned with complement-fixation, precipitin and skin tests, the serum-neutralization test as applied to convalescent and so-called "normal" human serum, and an *in vivo* protection test in monkeys. In most of this work purified and concentrated virus preparations were used, and in some of it we applied a method worked out for the purification of the virus-neutralizing fraction of serum.

COMPLEMENT-FIXATION AND PRECIPITIN TESTS

Neustaedter and Banzhaf (1) in 1917 reported positive complement-fixation reactions with 50% of the spinal fluids from forty-two frank and suspected cases of poliomyelitis, using trypsinized Berkefeld filtrates of poliomyelitis monkey cords as antigens. The serums of two patients failed to give positive results with the same antigens, and the spinal fluids of patients suffering from other diseases seldom gave positive reactions. Streptococci, tubercle bacilli, and meningococci, as well as Wassermann antigens used as controls, gave negative results. Kolmer and Freese (2) obtained positive fixation results with 6-16% of the spinal fluids from 130 frank cases of the disease, using aqueous extracts of spinal cords of fatal cases as antigens, and with 15-17% of the spinal fluids, using aqueous extracts of spleens and livers as antigens. Parallel tests were run with alcoholic extracts of poliomyelitis tissues and with aqueous extracts of

spleens and livers of cadavers normal with respect to poliomyelitis. The alcoholic extracts gave uniformly negative results, but the aqueous extracts of normal spleens and livers gave 5-7% positive results. These workers also reported some positive reactions with the serums of patients and aqueous antigens.

It should be pointed out that the distribution of the complement-fixing antibodies between the spinal fluid and the serum, as found by these investigators, is just the reverse of the distribution of the virus-neutralizing substances reported by Flexner, Clark and Amoss (3) and Flexner and Amoss (4). This incompatibility in distribution of the two prompted Schultz, Gebhardt and Bullock to suggest that the positive results which may be obtained with spinal fluids probably do not rest on an interaction between a specific antibody and the virus, but on some non-specific reaction.

Entirely negative results with complement-fixation tests have been reported by Wollstein (5), Gay and Lucas (6), Römer and Joseph (7) and Schultz, Gebhardt and Bullock (8). Wollstein studied the blood serum of poliomyelitis patients and convalescents, using as antigens aqueous extracts of poliomyelitis spinal cord and medulla and the spinal fluids of patients. Gay and Lucas tested the serums and spinal fluids of ill and convalescent persons against aqueous and phenolized extracts of poliomyelitis monkey cords. Römer and Joseph studied the spinal fluids of patients and convalescent human beings and monkeys with aqueous and antiformin extracts of the spinal cords of diseased monkeys. They also reported negative results with similar antigens and the serum of a monkey that had been hyperimmunized and had well-defined virus-neutralizing substances. Schultz, Gebhardt and Bullock inquired into the problem and obtained uniformly negative results

with the serums of convalescent and hyperimmunized monkeys which had been shown to have a fairly high titre of serum-neutralizing substances. They used as antigens collodion membrane filtrates of trypsinized cord suspensions, paper filtrates of aqueous suspensions of cord, and the ether and aqueous fractions of ether-treated cord suspensions of normal and poliomyelitis monkeys. These workers observed what at first appeared to be positive complement-fixation with some of these antigens when tested against the serum of rabbits which had been injected with poliomyelitis monkey cords, but the reactions proved to be non-specific for the virus when the same serums were tested against virus-free cord preparations.

The precipitin test was studied by Schultz, Gebhardt and Bullock. A variety of immune serums were tested with paper and collodion membrane filtrates and concentrated ether-treated virus preparations as antigens. These preparations were incubated either overnight in the ice box, two hours at 56° or two hours at 37° C. No evidence whatever of antibodies specific for the virus was elicited, although the serums were known to be virus-neutralizing and the virus known to be infectious.

It thus appears that neither complement-fixation nor precipitin tests are applicable to experimental poliomyelitis, whether or not positive results may be obtained with fresh materials from human beings. However, the problem in general is of such great importance that we undertook an investigation which particularly concerned the use of purified and concentrated virus preparations as antigens. It was thought possible that the preponderance of animal tissue proteins present in the ordinary antigens which had been used by others might be so great as to obscure any chance of the virus getting in contact with the complement and antibodies

in amounts sufficient to allow for a specific reaction to occur. In other words, a situation might exist which is similar to that discussed by Wells (9) as "concurrence of antigens." The chief justification, however, for this method of approach is the fact that it was noted in some preliminary experiments that crude virus preparations were likely to be anticomplementary in the strengths ordinarily used to infect monkeys, and that to get well beyond this anticomplementary phase it was necessary to dilute the virus into a zone of uncertain strength.

Experimental Work

A number of combinations of different antigen and serum preparations were studied under varying conditions of time and temperature of incubation in both complement-fixation and precipitin tests. In the former, the doses of complement used were also varied in accordance with the findings of Havens and Frank (10) that the test could be rendered more sensitive by using fractional units of complement. The antigens used were both crude and filtered suspensions of virus-cord tissue, some of which had been frozen and thawed repeatedly, and others frozen and dried, and virus suspensions which had been purified and concentrated to some extent by treatment with ether, alumina-gel, trypsin, and *in vacuo* distillation. The serums were from convalescent human beings and monkeys, hyperimmunized monkeys, and rabbits which had been injected with crude and purified virus suspensions. Some of these serums were treated with alumina-gel to effect some degree of purification. The method of preparation of each of these is given below.

Antigens

Untreated virus suspensions.--The cords of two or more

monkeys were taken at the height of poliomyelitis, pooled, ground, suspended in saline or distilled water, centrifuged and decanted. The supernatants were diluted so as to contain approximately 5%, 2.5%, 1%, 0.5%, and so on, by weight, of fresh spinal cord tissue. Concentrated NaCl solution was added if necessary to make the preparations isotonic. In each test a series of dilutions of antigens was used without serum, as well as with the test and control serums.

Filtered suspensions.--Seitz and Berkefeld N filtrates of both saline and distilled water extracts of pooled virus-cord tissue were used in dilutions comparable to the crude virus preparations.

Frozen and thawed suspensions.--Crude saline and distilled water emulsions of virus-cord tissue were rapidly frozen in a CO_2-ice-alcohol mixture and thawed in lukewarm water eleven successive times. This material was shown to be infectious for monkeys on the one occasion when tested. The reason for this procedure was to break down the tissue cells as far as possible by mechanical means, thereby liberating a greater amount of virus into the suspending fluid.

Frozen and dried preparations.--Crude and filtered emulsions of virus-cord tissue in distilled water and saline were dried *in vacuo* from a frozen state, sealed and stored in suitable tubes. Virulence-for-monkey tests were run on filtered and unfiltered preparations after two years' storage. Neither was infectious.

Ether-treated preparations.--Crude 10% emulsions of fresh virus-cord tissue in alkaline buffer solutions were shaken for approximately one hour with an equal volume of ether and allowed

to stand in the refrigerator overnight. These mixtures were centrifuged until a distinct three-layer separation occurred. The lower water-clear portions were collected and boiled *in vacuo*. In some instances this fraction was used in this and higher dilutions as antigens, in others it was concentrated ten-fold by boiling *in vacuo* at 30° to 37° C. and dialysed in collodion sacs against running distilled water. These preparations have proved to be uniformly infectious for monkeys. The method is that of Clifton, Schultz and Gebhardt (11).

Alumina-gel-treated preparations.--Unconcentrated ether-treated virus suspensions were brought to pH 6.5 by the addition of diacid phosphate solution, added to an equal volume of alumina-gel (Type C. Willstätter, standardized to contain approximately 22 mgm. of $Al(OH)_3$ per cc.), shaken for twenty minutes, refrigerated, centrifuged and decanted. The gel sediments were resuspended in isotonic phosphate solutions of pH 7.4, shaken for twenty minutes, and decanted. The supernatants were used as antigens without further treatment in some cases; in others, they were concentrated by boiling *in vacuo* and dialysed against running water. Such preparations have been regularly infectious for monkeys. This method of virus purification was originally described by Sabin (12).

Trypsinized virus suspensions.--Two methods, essentially the same in procedure but using different types of virus suspensions, were employed here. Fairchild and Foster trypsin was suspended in distilled water, passed through Seitz or Berkefeld N filters, and boiled *in vacuo* until it compared in turbidity to a 50% suspension of the original trypsin. This was stored aseptically in the ice chest. Crude 10% emulsions of fresh or glycero-

lated poliomyelitis monkey cords, some of which had been frozen and thawed repeatedly, were treated with sufficient sterile trypsin emulsion to make a final 2% concentration of the enzyme. These mixtures were rotated in an improvised machine in the incubator for periods up to twenty-four hours, then dialysed against running water.

A similar procedure was followed in treating the in vacuo concentrates of alumina-gel eluates of the virus. In each case the suspensions were buffered at pH 7.5-8.0 with NaH_2CO_3 or Na_2HPO_4. In the earlier experiments the Sorensen method of titrating amino acids was followed in an attempt to estimate the amount of digestion; this was discarded eventually, however, in favor of the qualitative bromine test for histidine. In each instance histidine was detected after digestion but not after dialysis nor before trypsin treatment. No parallel control tests for histidine were run on the trypsin alone; thus it is barely possible that the histidine was a product of digestion of the tissue vehicle of the trypsin and not of the cord protein. Virulence-for-monkey tests were run on these digests of the concentrated alumina-gel eluates. They were uniformly infectious, but some of the virus had been lost in the process, as was determined by biological assay of one of the preparations.

Serums

Convalescent monkey serum.--The serums of several monkeys that had survived a typical attack of poliomyelitis were tested from time to time for complement-fixing and precipitin antibodies.

Convalescent human serum.--The serums of fourteen children from the Orthopedic unit of Billings Hospital were obtained and tested for complement-fixing antibodies only. Six of these

individuals were receiving surgical corrective treatment for disabilities due to poliomyelitis; the age distribution was from seven to seventeen years. The others gave no history of poliomyelitis.

Hyperimmunized monkey serum.--The serum of a monkey that had received a long and severe course of injections of poliomyelitis monkey virus, and the serum of a monkey that had been treated similarly with normal monkey central-nervous-system tissue, were collected and tested for virus-neutralizing, precipitin and complement-fixing antibodies against various antigens.

Rabbit serum.--The serums of rabbits which had received intravenous, intraperitoneal and intradermal injections of crude and trypsinized crude virus were tested for complement-fixing antibodies. Control tests were run on the serums of rabbits which had been similarly treated with normal monkey cord tissue.

Alumina-gel-treated serum.--The virus-neutralizing fractions of pooled human convalescent serum and of pooled convalescent monkey serum, purified to some extent by the method described in a later section of this paper, were tested for complement-fixing antibodies.

Method of Conducting the Tests

Complement-fixation.--A 3 cc. test was employed, using 0.5 cc. of saline; 0.5 cc. respectively of 50%,* 40%, 20%, 10%, 5%, 0.5%, 0.1%, 0.01% and 0.001%, of the ether, alumina-gel, and trypsin-treated virus-cord suspensions, and 5%, 2.5%, 1%, 0.5%, 0.1%, 0.01%, 0.001%, and in some cases 0.00001% and 0.0000001%,

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*Percentage designations of virus strength are more or less questionable terms in any case. The meaning here is that so many grams of virus-cord-tissue are represented in the suspension, not accounting for losses which might have and probably did occur in handling.

of the crude, filtered, frozen and thawed, and frozen and dried virus preparations as antigens; 0.5 cc. of pooled fresh guinea pig serums containing two units, and in some cases one and one-half and one unit, of complement; 0.5 cc. of 1-5 dilutions of various serums which had been inactivated by heating at 56° C. for fifteen minutes; 0.5 cc. of hemolysin; and 0.5 cc. of a 2% suspension of sheep red blood cells. Primary incubation periods ranged from thirty minutes to four hours at 37° C. to eight to twenty-four hours at 8° C., and in some instances combinations of the two temperatures were used; however, the usual incubation was sixteen to twenty hours at 8° C. Secondary incubations were from thirty to ninety minutes at 37° C. for the first readings, with an additional eight to twelve hours at 8° C. for the second reading.

Precipitin tests.--Five-tenths cc. of both an aqueous and a concentrated aqueous fraction of an ether-treated virus-cord preparation were used in the serial dilutions, 50%, 25%, 10%, 5%, 2.5% and 1% as antigens (these antigens were water-clear), against pooled convalescent human, pooled convalescent monkey, pooled normal monkey, and hyperimmunized monkey serums in dilutions of 1-0, 1-5, 1-10, 1-25 and 1-50. These were incubated at 22° C., 37° C., and 55° C. for two days, with readings after intervals of 2, 4, 8, 16, 24 and 48 hours. Drying of the fluids was prevented by stoppering the tubes.

Results

Precipitin tests.--No reaction of any kind occurred in any of the tests which could be interpreted as a specific antigen-antibody reaction. All of the mixtures of the lower dilutions of serums and antigens jelled after a few hours' incubation at the

highest temperature. This was attributed to heat coagulation of the proteins.

Complement-fixation tests.--The results of these tests were likewise negative under all conditions. The anticomplementary property was very evident in all of the crude antigen preparations in concentrations greater than 0.5% of virus-cord suspension. This was greatly diminished, however, in the purified antigens; the ten-fold concentrates of the ether-treated virus, i. e., the 50% ether-treated concentrates, were quite anticomplementary, even after dilution to 40%, but were not anticomplementary in 20% dilutions. The alumina-gel eluates, some of which had been trypsinized, were only slightly anticomplementary in 50% concentrations, and not at all anticomplementary in higher dilutions. This is illustrated in Table I.

TABLE I

NON-SPECIFIC FIXATION OF COMPLEMENT BY VARIOUS ANTIGENS

Average antigen	Per cent concentration									
	50	40	20	10	5	2.5	1	0.5	0.1	0.05
Crude virus emulsions					++++	+++	++	±	-	-
Ether-treated virus					-	-	-	-	-	-
Al-gel eluate					-	-	-	-	-	-
Concentrated ether-treated virus	++++	++	-	-	-	-	-	-	-	-
Concentrated Al-gel eluate	+	-	-	-	-	-	-	-	-	-
Trypsinized concentrate of Al-gel	+	-	-	-	-	-	-	-	-	-

The serums of rabbits which had been immunized with crude and trypsinized crude virus and normal cord tissues fixed complement in the presence of all antigens against which they were tested. No attempt was made to run the titres of this antibody, as it was considered specific for the proteins of the cord tissue and in no way related to the virus.

Discussion

In presenting these observations attention should be invited to the fact that no difficulty whatever was experienced in demonstrating viable virus in most of the antigens used in the tests, and that the purified and concentrated antigens were markedly less anticomplementary than the crude virus preparations. It should also be mentioned that the convalescent and hyperimmunized monkey serums and at least some of the convalescent human serums were in all probability virus-neutralizing, although tests were not run to determine this in all cases.

The antigens were, in many cases, we believe, higher in virus content than any that have been employed in poliomyelitis complement-fixation tests before. However, it is problematical just how much virus was present in the various concentrated and purified suspensions. Sabin reported that there was some loss in virus when purified by the alumina-gel method, as evidenced by the fact that second eluates of the virus-gel complex were infectious; nevertheless the loss could not have been of major importance because the first eluate was infectious in almost as high dilutions as was the original untreated virus preparation. The question arises here regarding the accuracy of virulence-for-monkey tests, and it is apparent that no great dependence can be placed on this technique, especially in view of the very recent work of Theiler

and Bauer (13), in which it was shown that monkeys vary greatly as individuals in response to relatively small doses of virus.

It is very possible that the methods employed in treating the virus effected much less purification and concentration of the virus than we have supposed, and we feel quite sure that the preparations designated as 50% virus concentrates did not contain ten times as much virus as the original 5% aqueous emulsions from which they were derived. On the other hand, it is quite certain that some concentration of the virus was effected, if only in relation to the fats and some of the cord proteins which were removed.

It seems logical to suppose that if there were complement-fixing or precipitin antibodies present in recovered monkeys and persons, this would have been apparent in some of these tests. It may be that these antibodies are present in very low dilution, and will become apparent only when serums of very high titres are prepared or when the fraction is concentrated and purified by laboratory methods.

IN VIVO PROTECTION TESTS

There are numerous instances in the literature of irregularities in results that appear in applying the serum-neutralization test in poliomyelitis. It has been the experience of many that animals inoculated with similar mixtures of serum and virus will vary in their reaction, some going through a typical attack of poliomyelitis, others not reacting in any way. This phenomenon is not of course confined to poliomyelitis. On the other hand, it is well recognized that individuals of almost every animal species vary in their susceptibility to infectious agents; Topley and Wilson (23) discuss this amply under the heading "the measurement of virulence" and show that tests involving animals must be applied in large numbers before the results may be considered valid. This is well nigh impossible in poliomyelitis, however, because of the tremendous outlay it requires, and consequently no dependence can be placed on the results of the serum-neutralization test in individual cases.

A test for the activity of the serum toward the virus, independent of variation in animal susceptibility, might be arrived at if in single animals one could protect a section of the central nervous system tissue against the virus, while lesions are developing in another portion, by the interposition of antibody containing serum. Of course, such a mechanism, if worked out, might present difficulties more serious than those now existent in the serum-neutralization test; on the other hand, the situation is already acute and almost any sort of departure from

the old and proved methods is justifiable. And indeed, this type of experimentation is not unprecedented, for similar effects of tetanus antitoxin in rabbits and cats were early shown by Marie and Morax, and Meyer and Ransom (24).

The observations of Jungeblut (25) and Hurst (26) offered an opportunity to study the relationship of the serum substance to the virus in the manner already alluded to. Jungeblut found that in monkeys the virus could be prevented from migrating from the brain (the site of inoculation) and the cervical cord to the lumbar cord by surgical neuro-transection. Hurst similarly reported that an entrance of the virus into the cord could be prevented by sectioning the sciatic nerve at a point proximal to the site of inoculation of the virus.

It was reasoned that if the virus does travel in the nerve tissue proper and if section prevents its further progress and if the production of serum-neutralizing substances in the disease is to be considered a specific defense mechanism, then one might expect to render a section of the nerve tissue impassable to the virus by saturating it with the virus-neutralizing substance. Two objections to this type of experimentation are immediately apparent. In the first place, does the virus travel only along the nerve tracts of the CNS or may it "by pass" through the sympathetic nerves, the lymph channels, or the blood stream? In the second place, can any section of nervous tissue be kept saturated with serum until the virus in a nearby section of tissue is introduced, passes through its stages of increase in amount and disease production, and disappears? The first objection cannot be obviated on a basis of fact; however, most of the recorded experience to date indicates that conditions may be set up, especially along the sciatic trunk, in which only by the nerve proper can a sufi-

cient amount of virus travel into the CNS to produce the disease. This evidence may be listed briefly as follows: scant records of isolation of virus from the blood stream and cerebrospinal fluid, as compared with numerous isolations from CNS tissue; infrequent infections following intravenous inoculations, as compared with a very high proportion of infections with intracerebral inoculations; and the reported inability of the virus to pass a surgical section of the sciatic nerve and of the spinal cord, as well as the inability of workers to infect monkeys by intranasal virus instillation following section of the olfactory nerve trunk, as compared with the extreme frequency of infection by intranasal instillation of virus in normal monkeys. There is no precedent upon which to discuss the second objection, although one might reasonably expect to saturate fairly well a portion of the spinal cord, or better, a section of the sciatic nerve, with serum by multiple injections under pressure; the question of how long the serum antibodies will remain in an area can be determined only by experimentation.

Experimental Work

Two types of experiments were undertaken in an effort to demonstrate some *in vivo* virucidal effect of serum along the line indicated above. The first consisted in inoculating convalescent human serum in the thoracic portion of the spinal cord at arbitrary periods following the inoculation of virus intracerebrally. In the second type of experiment, convalescent human serum was inoculated in the gluteal region of the sciatic, proximal to the site of virus inoculation.

Intra-cord-tissue serum inoculation.--In the first experiment of this part of the investigation three monkeys were inocu-

lated intracerebrally with 1.0 cc. of 1% virus-cord; on the following day the spinal cord of one of the monkeys was exposed by sectioning the neural arch of the second and third thoracic vertebrae through the left and right posterior neurapophyses and lifting out the interposed portion of the laminae. A small hole was accidentally opened in the dura mater but only a small amount of spinal fluid was lost during the operation. As large an area of the cord as could be reached with a small bore needle was treated with 1.5 cc. of pooled convalescent human serum under considerable pressure. The second and third monkeys were similarly treated on the second and third days, respectively, after virus inoculation. All developed typical poliomyelitis with paralysis of all extremities in the usual period. Sections of the cord showed poliomyelitis lesions throughout.

The second experiment differed from the first in the following respects: serum injection was accomplished by inoculating between the laminae after removal of the posterior spinous processes, sufficient latitude being realized to enable all parts of the cord to be reached, except that immediately under the laminae. One cc. of serum was injected one, two, four and five days after virus inoculation. All four of the monkeys so treated developed complete flaccid paralysis of all extremities in the usual time. Sections of the cord showed poliomyelitis lesions throughout. No bacterial infections were noted at autopsy.

These results may be seen in Table II.

TABLE II

INTRAMEDULLARY INJECTION OF CONVALESCENT HUMAN SERUM

Monkey Number	Date of Intracerebral Injection of Virus	Intramedullary Injection of Serum		Result
		Amount	Date or Dates	
1	Aug. 13	1.5 cc.	Aug. 14	Aug. 20 Complete flaccid paralysis of all extremities, poliomyelitis
2	Aug. 13	1.5 cc.	Aug. 15	Aug. 22 "
3	Aug. 13	1.5 cc.	Aug. 16	Aug. 20 "
4	Aug. 25	1.0 cc.	Aug. 26,27,29,30	Sept. 2 "
5	Aug. 25	1.0 cc.	Aug. 26,27,29,30	Sept. 1 "
6	Aug. 25	1.0 cc.	Aug. 26,27,29,30	Sept. 1 "
7	Aug. 25	1.0 cc.	Aug. 26,27,29,30	Sept. 2 "

Sciatic inoculations.--Similar experiments were carried out with sciatic inoculations of serum and virus in which the problem was to determine whether or not the virus could be prevented from migrating into the principal part of the CNS by saturating an intermediate area of the nerve with serum antibodies. Hurst had already reported uniform success in infecting monkeys by intra-sciatic inoculation. The first experiment was as follows.

Experiment 1. One monkey was inoculated with 0.5 cc. of

purified virus in the popliteal region of the left sciatic nerve, and with 1.5 cc. of an antibody extract of pooled human convalescent serum in the coccygeal region of the same nerve. Thirty-four days later this animal received 0.5 cc. of purified virus in the popliteal section of the right sciatic. Daily observations were made from the day of the first inoculation until the twenty-eighth day after the second inoculation. No symptoms of poliomyelitis were noted during this period. A subsequent intracerebral virus inoculation produced typical poliomyelitis in this animal, showing that it had at least no solid immunity.

Experiment 2. A second experiment was undertaken, using three monkeys, and was identical with the first, except that 0.6 cc. of purified virus and 1.0 cc. of antibody extract were used instead of 0.5 cc. of virus and 1.5 cc. of serum. Greater precaution was taken to insure trauma of the nerve and pressure inoculation. Forty-four days later these animals were reinoculated with 0.5 cc. of purified virus in the popliteal region of the right sciatic. No symptoms of poliomyelitis were noted in any of these monkeys at any time during the experiment. Subsequent intracerebral virus inoculations brought these monkeys down with poliomyelitis. It should be mentioned that the virus used in the first inoculation of these monkeys was not infectious by the intracerebral route in one test animal.

Experiment 3. At this point the method of attack was changed for the reason that it was held possible, though not probable, that the first inoculations, consisting of both serum and virus, were producing enough immunity in the lower portion of the spinal cord to prevent the migration of virus into the principal part of the CNS from the previously uninoculated sciatic.

Three new monkeys and one which had previously received

an intracerebral inoculation to test for the presence of virus in a rat brain, were inoculated in the popliteal section of the left sciatic with 0.5 cc. of purified virus. No symptoms were observed in these animals in the twenty-three days of observation, although the intracerebrally inoculated control animal showed the virus to be potent.

Experiment 4. In spite of the failure of the previous experiments, another experiment was performed, using both serum and virus. In this experiment the four monkeys from the previous experiment (Experiment 3) were inoculated with 0.5 cc. of 1% crude virus-cord suspension in the sacral region of the right sciatic just distal to the site which was simultaneously inoculated with 0.5 cc. of raw pooled convalescent human serum. Three of these monkeys remained well through the period of observation; the fourth had a rise in temperature seventeen days after inoculation and showed complete flaccid paralysis of the legs the following day. The serum and virus both were shown to be potent in control animals. Two of the survivors of this experiment were eventually tested for resistance to the virus by intracerebral inoculations. Both proved to be susceptible.

Experiment 5. Again it was decided to retest the efficacy of the method of virus inoculation. The three survivors of the previous experiment were inoculated in the sacral region of the right sciatic with 0.5 cc. of 10% crude virus. All remained well during the following twenty-eight days in spite of a proved potency of the virus. One of these animals was transferred to non-poliomyelitis work and the other two succumbed to small doses of intracerebrally inoculated virus.

These results may be seen in Tables III and IV.

TABLE III

SIMULTANEOUS INTRASCIATIC INOCULATIONS OF SERUM AND VIRUS

Monkey Number	Virus Inoculation	Serum Inoculation	Result	Virus Control
8	0.5 cc. purified virus in popliteal portion of left sciatic nerve	1.5 cc. purified serum antibody in gluteal portion of left sciatic nerve	No poliomyelitis	Poliomyelitis
9	"	1.0 cc. purified serum antibody in gluteal portion of left sciatic nerve	No poliomyelitis	No poliomyelitis
10	"	"	"	"
11	"	"	"	"

REINOCULATION OF ANIMALS WITH VIRUS IN THE SCIATIC NERVE

Monkey Number	Virus Inoculum	Result	Virus Control	Subsequent History of Animal
8	0.5 cc. purified virus in popliteal portion of right sciatic nerve	No poliomyelitis	Poliomyelitis	Succumbed to intracerebral injection of poliomyelitis virus
9	"	"	"	Died of intercurrent infection
10	"	"	"	Succumbed to intracerebral injection of poliomyelitis virus
11	"	"	"	"

TABLE IV

SIMULTANEOUS INTRASCIATIC INOCULATIONS OF SERUM AND VIRUS

Monkey Number	Virus Inoculation	Serum Inoculation	Result	Virus Control
12	0.5 cc. of virus in gluteal portion of right sciatic nerve	0.5 cc. of serum in gluteal portion of right sciatic nerve proximal to site of virus inoculation	No poliomyelitis	Poliomyelitis
13	"	"	"	"
14	"	"	Poliomyelitis	"
15	"	"	No poliomyelitis	"

REINOCULATION OF ANIMALS WITH VIRUS IN THE SCIATIC NERVE

Monkey Number	Virus Inoculation	Result	Virus Control	Subsequent History of Animal
12	0.5 cc. of virus in gluteal portion of left sciatic nerve	No poliomyelitis	Poliomyelitis	Succumbed to intracerebral injection of virus
13	"	"	"	Not tested
15	"	"	"	Succumbed to intracerebral inoculation of virus

Discussion

The most interesting result of these experiments is the complete failure of the virus-neutralizing serum to inhibit the migration of the virus from one part of the CNS tissue to another. This was particularly striking in the case of the brain-cord inoculations. Here there was no evidence of a delay in infection of the lumbar cord in spite of the administration of 4 cc. of potent serum over at least 3 cms. of the thoracic cord over a period of four days. It was suggested in the opening of this paper that in case of a negative result for the serum certain objections might be raised. The objection that the virus had "by passed" the serum block still holds, but this might itself be objected to on the basis of Jungeblut's and Hurst's observations that surgical section prevented migration of the virus. The criticism that the cord may not have been saturated in all parts (of the treated area) at all times cannot be obviated; it is certainly well within the range of probability that the antibodies did not diffuse or otherwise get into every axis-cylinder down which the virus might have travelled. Nevertheless, it seems that at least in one of the experiments this section of the cord must have contained a high concentration of virus-neutralizing substance from soon after the virus inoculation until death of the animals. One might reasonably expect a much less intense pathologic reaction in the lumbar section of the cord if as many as half of the virus paths were blocked out, but no difference was noted in sections of the cord enlargements taken at autopsy.

The failure to realize infection after intrasciatic inoculation of virus is inexplicable, especially in view of the claims of uniform success with this type of experimentation by

Hurst. However, a point of interest here is the fact that in one monkey typical poliomyelitis developed after intra-sciatic inoculation of the virus, in spite of a proximal serum inoculation. There is a bare possibility that this take was from the previous experiment, in which virus alone was inoculated in the other sciatic, although such a thing is highly improbable, as forty days had elapsed from the time of this inoculation until the onset of paralysis. Here again the objections listed for the results in the brain-cord experiments may be raised.

The results of these experiments are in accord with those obtained in other protection tests in poliomyelitis which have been performed by a number of workers. These have been very recently analysed and summarized by Harmon (27), who concludes that there is no significant difference in the outcome in monkeys which have received prophylactic and therapeutic serum treatment and those which have not.

Summary

Seven monkeys were given intracerebral doses of poliomyelitis virus and subsequent intra-cord doses of virus-neutralizing serum. All succumbed to typical poliomyelitis with complete flaccid paralysis of all extremities.

Nineteen sciatic inoculations of virus were made at different intervals among a group of eight monkeys. Eight of these nineteen virus inoculations were accompanied with proximal inoculations of convalescent human serum. One of these latter tests resulted positively in typical poliomyelitis; the others were negative. The resistance of most of the animals was tested and they proved to be susceptible to the virus by subsequent intracerebral inoculation.

AN ANALYSIS OF THE SERUM-NEUTRALIZATION TEST

The nature of the virus-neutralizing substance which appears in the serum of persons and animals upon recovery from an attack of poliomyelitis and which is found in the majority of adult, so-called "normal" persons, is problematical. This substance has already been considered in the light of its complement-fixing, precipitin and protective qualities, and as yet these properties have not been demonstrated. Schultz, Gebhardt and Bullock (8) suggested that it is similar in some respects to the antitoxins and speculated that the serum-neutralization reaction is essentially of the toxin-antitoxin relationship. Jungeblut and others (21) proposed that the substance is a non-specific fraction or property of serum which is largely dependent for its appearance in the body upon physiological maturation and not entirely on exposure to the virus. Their theory is more or less hypothetical and rests principally on the experience of many poliomyelitis workers that this neutralizing property occurs usually only in low dilution, and indeed may even be absent, in the serum of convalescent monkeys and human beings in spite of a solid immunity; that it occurs in a large proportion of adults who supposedly have been in contact with neither the disease nor, perhaps, even the virus; that it is usually produced only with difficulty in refractory animals; and that it is therapeutically ineffective in the experimental disease. Jungeblut has carried on an investigation along this line in the last few years and has reported additional evidence to support his contentions, some of

which has been contested by others (29). Should the "maturation" theory be proved, that portion of the epidemiology of the disease which rests upon the results of this test will be rendered open to question.

In view of the impractical and unreliable status and questionable specificity of the serum-neutralization test, we have undertaken a series of experiments which were designed to throw some light on the nature of the protective antibody of convalescent human serum and its relationship to the virus and to the antibody of so-called "normal" human serum. Our first steps have been to work out a method for the purification of the neutralizing substance and to make certain observations on its behaviour toward the virus.

Only one method of antibody purification has hitherto been used in poliomyelitis investigations. Weyer, Park and Banzhaf (30), and Fairbrother and Morgan (31) employed the salt fractionation technique to concentrate and purify, to some extent, the antibodies of hyperimmunized horse serum. Since these two groups of workers disagree as to what protein fraction of the serum, pseudoglobulin or euglobulin, contains the higher concentration of the antibody, and since the method for other reasons was unsuited to this investigation, our attempts at antibody purification have been along other lines. Undoubtedly, the best means of purification would be the recovery of the antibody from a pure form of virus-antibody complex. Inasmuch as Olitsky, Rhoads and Long (32), and Schultz, Gebhardt and Bullock (8) have shown that the virus may be recovered from neutral serum-virus mixtures, it seemed that the antibody might be recoverable from such a complex. The difficulty in this method is in isolating the complex from solution, for it does not occur in solid form. However, Sabin's

(12) report of the adsorption of the virus to alumina-gel offered what seemed to be possibly a means of separation, as it was thought that perhaps the antibody could be caused to combine with the virus which was already adsorbed to gel, that is, with a virus-gel complex.

In addition to the attempts to adsorb the antibody specifically to the virus of the virus-gel complex, efforts have been made to purify it to some extent by treating it with alumina-gel in much the same manner that Sabin adsorbed and eluted the virus. Numerous investigators (33, 34), in studying the behavior of antibodies toward adsorptive agents, have found that antibacteria, antitoxin, antivenom, and antivirus serum substances may be adsorbed to powdered forms of siliceous earths, charcoals, chalks and other substances. Most of them observed that while dissociation of the complexes often occurs readily in the animal body, it is difficult to induce the same effect in the test tube. Frankel and Olitzki and others (35) have recently reported that Bact. typhosum agglutinins and diphtheria antitoxin could be dissociated from kaolin complexes by glycine. The amount of antibody recovered by this method is only 25% of the original serum antibodies.

We felt justified in undertaking this investigation because either the perfection of another means of antibody purification or the production of a complex consisting of virus and some inert microscopic material, capable of adsorbing the antibody specifically, would be a valuable adjunct to poliomyelitis investigation. For instance, such a virus-adsorbent complex might be used as an antigen for tests such as agglutination or phagocytosis, or it might serve as an agent for detecting the presence of strain-specific virus-antibodies by the ordinary ab-

sorption technique, and thereby show some difference in the antibodies of normal and convalescent persons. Furthermore, another method of purifying the antibody might be of some practical value in therapy.

Experimental Work

The scope of this work has been limited to some extent by the care and cost involved in monkey work. However, by using animals which had been previously inoculated with innocuous serum-virus mixtures in some of the work, more experiments have been undertaken than might have been otherwise possible. Some investigators are opposed to using monkeys which have survived one inoculation for other experiments of the same type, fearing that the first inoculation, although producing no symptoms of poliomyelitis, may have altered the normal level of susceptibility of the animal. The majority, however, feel that if any immunity is produced in this way it is not appreciable. We have not encountered a greater number of irregularities attributable to individual resistance in the experiments with used animals, than in those with unused monkeys. Furthermore, by using duplicate monkeys in testing most of the material our results may be considered to be more accurately determined.

Preliminary experiments.--At the outset of this work it was thought advisable to determine whether or not the virucidal fraction of convalescent human serum could be absorbed* by small blocks of virus-cord tissue. For this purpose the following ex-

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*The terms adsorb and absorb and their derivatives are often used interchangeably, but in the present paper they signify two distinct things. Absorb is used in the sense of removal of and adsorb in the sense of adherence to; for example, absorbed serum, serum from which the antibody has been removed, and adsorbed antibody, or adsorbate, that which is collected on the adsorbing agent.

periment was performed.

Approximately 1 gm. each of virus-cord and normal cord were cut into small blocks measuring from 1 to 2 mm. to the side and were placed in tubes containing 4 cc. of a 1-5 dilution of convalescent human serum. The mixtures were rotated slowly in the incubator at 37° C. for two hours and then refrigerated overnight. The following morning the respective supernatant fluids from centrifuging these mixtures were collected, mixed with test virus and then inoculated intracerebrally into monkeys.

While the animal receiving test virus alone developed typical poliomyelitis, neither of the animals receiving the mixture of test virus and serum which had been exposed to virus-cord or normal cord blocks showed any symptoms of the disease during an extended period of observation.

These results indicated that little or no absorption of the antibody had taken place. Quite likely the absorption could be increased by decreasing the size of the virus-cord particles. This, however, would present extreme difficulties, because there is no means for removing the considerable amount of dispersed ultra-microscopic particles of virus (-cord?) which would undoubtedly result. Consequently we discarded this type of experiment.

In turning to the use of the virus-gel complex for absorption of serum, it seemed desirable to have the virus in a form more pure than the ordinary crude or filtered suspensions. We adopted immediately the method of ether treatment of virus-cord tissue described by Clifton, Schultz and Gebhardt (11), because it is simple and convenient to follow. This method is briefly as follows.

A given amount of virus-cord suspension is shaken for one

hour with an equal volume of ether and the resulting mixture is placed in a separatory funnel and stored in the refrigerator. Three layers appear; the lowest, which is water-clear and contains most or all of the virus, is collected and evacuated to remove the ether.

A preliminary experiment confirmed the authors' claims for the method. Two animals receiving intracerebral doses of the lower layer developed typical poliomyelitis in a very short time after inoculation. Although no biological tests were run on the other fractions, a physical inspection revealed that a large amount of lipoid and lipo-protein material, thought to be cell stroma and membranes, had been removed.

Further investigations with serum absorptions.--The failure to absorb antibodies from neutralizing serum as attempted in the previous experiment might be overcome, it was thought, by using an antigen in a finely divided state, which could be both dispersed and aggregated readily. Therefore, we began a series of experiments in which aluminum-hydroxide-gel, to which an ether-treated preparation of either virus or normal cord had been adsorbed, was used for serum absorption. Similarly, neutralizing human poliomyelitis serum was treated with gel alone with the purpose of adsorbing the immune substance to the same reagent. Convalescent and normal human serums obtained from The Deutsch Serum Center and the PMV strain of virus from The Rockefeller Institute were used throughout the experiments. The proportion of one part of a 1% suspension of virus-cord to three parts of serum was used in the neutralization tests on the various serum preparations.

Methods

Preparation of the aluminum-hydroxide-gel and antigens.--The alumina-gel was prepared according to the directions given by Willstätter (38) for "Type 'C' gel."

One and one-half liters of distilled water containing 500 gm. of $Al_2(SO_4)_3$ are added to 7.5 liters of 4% NH_4OH warmed to 70° C. After thirty minutes of vigorous stirring, the $Al(OH)_3$ which is formed is washed five times with distilled water, and then treated with four liters of 4% NH_4OH at a temperature of 70° C. The gel is washed again twelve times with distilled water. The final gel sediment is resuspended in a sufficient amount of pure water to make a standardized suspension containing about 25 mgm. per cc. of water. This material is sterilized by autoclaving.

We have slightly modified and combined the methods of Clifton, Schultz and Gebhardt, and Sabin for the purification of the virus to prepare the virus-gel complex. This procedure is as follows.

The supernatant fluid from centrifuging a 10% suspension of virus-cord in isotonic phosphate buffer solution of pH 7.4 is shaken for one hour with an equal volume of CP ether. After overnight refrigeration and after centrifuging, the lower water-clear virus-containing layer is collected and evacuated for twenty minutes, to remove the last traces of ether. This somewhat purified virus-cord suspension is brought to a pH of 6.5 by the addition of isotonic KH_2PO_4 solution, and is further diluted to make a 5% suspension by the addition of neutral isotonic NaCl solution. This final virus-cord suspension is added to the sediment of an equal volume of the standardized gel suspension, shaken for twenty

minutes, and then refrigerated overnight. After centrifuging the mixture, the heavy virus-gel sediment is collected and used as a serum absorbent. A small portion of the complex is resuspended in isotonic phosphate buffer solution of pH 7.4, shaken for twenty minutes, and then centrifuged. The supernatant fluid is collected and a portion of it is injected intracerebrally into monkeys to determine whether or not viable virus is present in the complex.

The normal-cord-gel complex for control purposes is prepared in exactly the same manner, except that normal monkey cord is used instead of poliomyelitis cord tissue.

Absorption of serum.--It is necessary to acidify the serum before adding it to the virus-gel complex because the virus does not remain adsorbed to the gel when the suspending fluid is slightly alkaline.

Acidification of the serum is accomplished by adding a sufficient amount of isotonic KH_2PO_4 solution to bring it to a pH of 6.5. Also, in order to have uniform conditions in serial experiments, the acidified serum is further diluted to 1-5 by the addition of chemically neutral isotonic NaCl solution. Potentiometric determinations of the hydrogen-ion concentration are made at various stages in the preparation of the serum.

A quantity of the 1-5 dilution of acidified serum is added to the sediment of an equal volume of either the original untreated gel, normal-cord-gel complex, or virus-gel complex suspension, as the case may be. (Since in the preparation of these complexes an equal volume of gel suspension and virus or normal cord suspensions are used, the volume of serum being absorbed is equal to the volume of 5% virus-cord or 5% normal-cord suspensions represented in the complexes. In one experiment an attempt

was made to double the amount of virus-cord and normal-cord in the complexes. Here the volume of virus represented in the complex was twice the volume of serum subsequently absorbed; thus, for example, 10 cc. of serum was absorbed by a complex containing the virus originally present in 20 cc. of a 5% suspension.) The tubes containing these materials are stoppered and rotated in the incubator at 37° C. for from two to three hours. After centrifuging, the supernatant fluids are collected and tested for their virucidal power. In order to trace the fate of the antibody, the adsorbates are eluted and tested. For this purpose the centrifugates of the gel and gel-complex mixtures with serum are resuspended in phosphate buffer solution of pH 7.4, agitated for a short time and then centrifuged. The resulting supernatant fluids are tested for virus or antibody, as the case may be.

The following figures, 1, 2 and 3, represent schematically the steps taken in the preparation of the antigens, the serum, and their absorbates and eluates, and indicate what materials were tested.

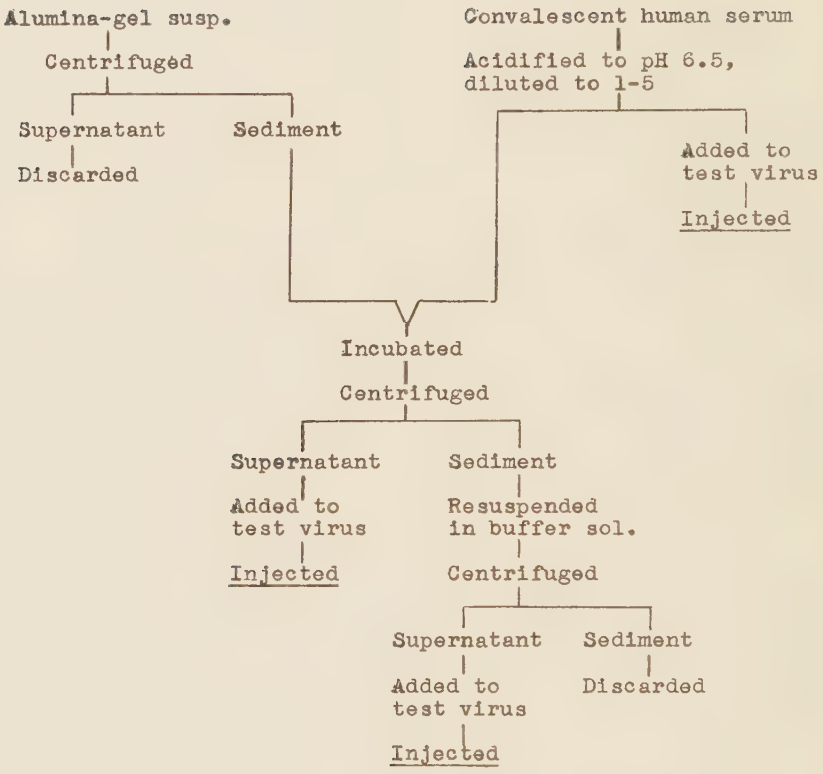

Figure 1.--Schematic representation of the absorption of serum with alumina-gel and the tests made on various fractions.

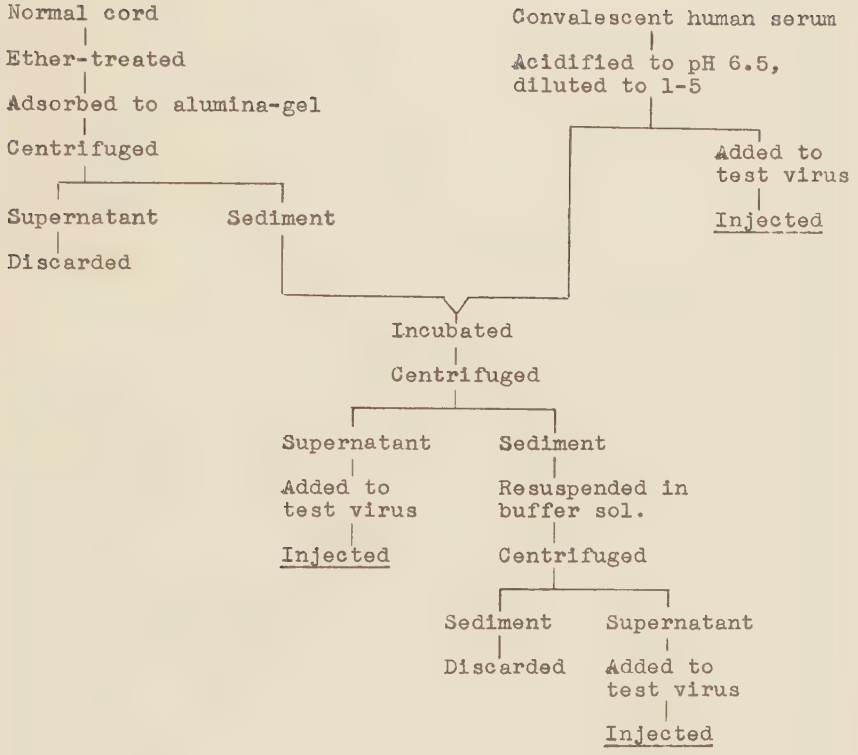

Figure 2.--Schematic representation of the absorption of serum with normal-cord-gel complex and the tests made on various fractions.

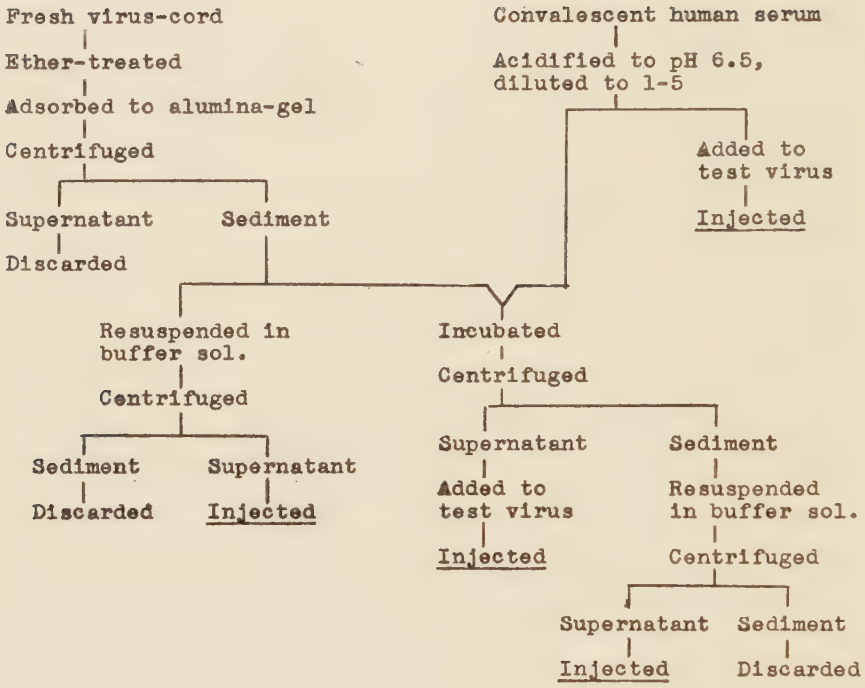

Figure 3.--Schematic representation of the absorption of serum with a virus-gel complex and tests made on various fractions.

Results

Tests on the antigens.--The presence of viable virus in the virus-gel complexes used for serum absorption has been determined by inoculating monkeys with eluates of the complexes. In six of the experiments all of the ten animals receiving the eluates died of typical poliomyelitis, while in two of the experiments neither of the animals treated with similar material showed symptoms of the disease in the period of observation. The results of this part of the experiment are listed in Column 1 of the Master Table. They are confirmatory of some of the findings made by Sabin in his work on the purification of the virus.

Experiments with convalescent human serum.--A. Absorbed with alumina-gel. Ten of the thirteen animals receiving incubated mixtures of immune serum which had previously been absorbed with alumina-gel, plus test virus, developed typical poliomyelitis. Two of these surviving animals were members of an experiment in which the virus used for testing may have been weaker than usual. In this experiment the test virus control animals suffered a light attack of the disease, and that only after a much delayed incubation period. The failure in the other animal cannot be accounted for except as an instance of biological variation on the part of either the test animal or the adsorbability of the antibody. Nevertheless, these variants will be considered in the final analysis. These experiments showed that the antibody was either inactivated by or adsorbed to the alumina-gel.

That the antibody was absorbed is more clearly shown by the results of the tests on the eluates of the gel adsorbates of the serum. In five experiments ten of the twelve monkeys receiving the eluates mixed with test virus survived without showing

any symptoms of the disease. In one of these experiments the eluate was titrated for its antibody content. In this instance, the antibody-gel complex was suspended in an amount of phosphate buffer solution equal in volume to one-half the volume of the serum originally absorbed. Samples of the untreated serum in dilutions of 1-5, 1-10, 1-25, and 1-50, and samples of the eluate in relative dilutions of 1-2.5, 1-5, 1-10, 1-25 and 1-50 were examined for the neutralizing substance and all neutralized the test dose of virus, indicating that not only was practically all of the antibody absorbed from the serum, but also that it was concentrated by this method. However, the failure to reach an end point here mitigates the results to some extent. A representation of the behaviour of the antibody toward alumina-gel may be seen in Figure 1, and the results of this phase of the experiments may be seen in Table V and in Columns 6 and 7 of the Master Table.

B. Absorbed with normal-cord-gel complex. The results of this part of the experiment are listed in Columns 4 and 5 of the Master Table. All of the four animals receiving mixtures of test virus and serum which had previously been absorbed with the normal-cord-gel complex developed typical poliomyelitis in the usual time after inoculation; that is, this type of complex removed the antibody from the serum. The fate of the neutralizing substance when thus treated was investigated further in one of the experiments. An eluate of the adsorbate protected two animals against test doses of viable virus, indicating that the antibody can be eluted from this complex, as was formerly demonstrated in the case of the gel adsorbates of serum. The behaviour of the antibody toward the normal-cord-gel complex is represented in Figure 2.

We feel that in these absorptions there must have been a

sufficient amount of gel which did not have normal cord attached to it, that is, free gel, to adsorb the antibody, because it is not likely that the normal cord tissue alone has an adsorptive capacity for the antibody, at least to the extent experienced here. In the second of these experiments an attempt was made to double the amount of cord tissue adsorbed to the gel, and the period during which these materials were shaken together was increased to three times what it was in the previous experiment in an effort to decrease the amount of free gel. This change, however, produced no difference in results. The question arises, Was any of the cord tissue adsorbed to the gel? A positive biuret test on the eluate showed that there was. The only alternatives to the explanation on the basis of the free gel are that perhaps the gel has adsorptive capacity for the antibody despite the presence of normal cord, or that the antibody is more readily adsorbed by the gel and replaces the tissue proteins.

C. Absorbed with the virus-gel complex. In the first experiment in which serum absorbed with the virus-gel complex was tested, one of the two animals receiving a mixture of test virus and the absorbed serum succumbed to poliomyelitis, while the other animal, although receiving material from the same tube, showed no symptoms of the disease. While this disagreement in result may be accounted for by the individual resistance and susceptibility of the test animals, it is entirely possible that the test virus was inactivated to a certain extent by the serum preparation, leaving it on the border line of infectivity.

In the second experiment a single animal was used to test a similar preparation. This monkey developed the characteristic high temperature and a slight tremor, both of which passed without any other symptom of poliomyelitis being exhibited. In a

test some time later, the serum of this animal showed no neutralizing property, which, however, does not mean that it could not have had an attack of the disease, for we have had in our laboratory an animal which showed no virucidal activity of serum even six months after a moderately severe attack of the disease.

In the third experiment on this phase of the problem, two animals succumbed to the inoculation of a similar mixture, indicating that the antibodies had been removed by the virus-gel complex. The virus of this complex was, however, shown to be innocuous by the survival of the control animal which received the eluate. The absence of virus in the complex would of course place this experiment in the group in which untreated gel or the normal-cord-gel complex was used as an absorbent. However, the failure of one animal to develop the disease upon injection does not necessarily mean that viable virus was not present in the inoculum; consequently, we cannot say that there was no virus present in the antigen in this experiment.

In the fourth experiment, two of the four monkeys injected with the same mixture of test virus and serum which had been absorbed with the virus-gel complex died of typical poliomyelitis, while the other two showed no symptoms of the disease. In the fifth and sixth experiments all of the four animals receiving similar preparations survived, making a total of eight survivors in thirteen monkeys treated with this type of preparation. It thus appears that human convalescent serum is irregularly freed of its antibody by absorption with the virus-gel complex.

The question arises, What happens to the virus during the absorption of serum with the virus-gel complex? The results of five experiments showed that it was usually inactivated during or after absorption of the serum. In the first two of these experi-

ments the four animals receiving the eluate of the virus-gel adsorbate of serum survived without having shown any symptoms of the disease. In the next three experiments the procedure was changed a little in that the eluates were inoculated immediately after elution, rather than storing them in the ice box overnight and then inoculating. One of five monkeys injected with these eluates died of typical poliomyelitis; the other four remained well throughout the subsequent twenty-eight day period of observation. It is entirely possible, of course, that with such relatively low dilutions of serum the effect of the antibody on the virus may take place after the mixture has been inoculated. On the other hand, it is equally possible, and perhaps more probable, that the neutralization in the present instances took place before inoculation.

These results may be seen in Table V and in Columns 2 and 3 of the Master Table, and a representation of the preparation of the serum fractions and the tests made on them appears in Figure 3.

Experiments with normal human serum.--A. Absorbed with alumina-gel. In five experiments twelve monkeys were inoculated with test virus and normal human serum which had been absorbed with alumina-gel. Six of these animals died of poliomyelitis and four remained well during the period of observation; the other two died of intercurrent disease and were withheld from consideration. In one of these experiments in which there were two survivors, an error was made in the preparation of the alumina-gel which may have accounted for its failure to absorb the antibodies to any great extent.

The eluates of these gel adsorbates usually neutralized the virus. Eight of twelve animals in this series of tests sur-

vived, three succumbed to poliomyelitis, and one died of intercurrent infection. It is interesting to note that each of the three animals developing poliomyelitis received material which was innocuous for duplicate animals. The results of these experiments may be seen in Table V and in Columns 11 and 12 of the Master Table.

B. Absorbed with the virus-gel complex. The results of these experiments were similar to those obtained in the same phase of the work with convalescent human serum. Twelve monkeys were inoculated with mixtures of test virus and normal serum which had been absorbed with the virus-gel complex. One of these animals died of intercurrent disease, two died of poliomyelitis, and the other nine remained well during the usual period of observation. Here again the two monkeys which developed poliomyelitis received injections which were innocuous for duplicate animals.

Of eight animals receiving the eluates of the virus-gel adsorbates of serum in three experiments, one died of poliomyelitis and the other seven remained well. These results may be seen in Table V and in Columns 9 and 10 of the Master Table.

Results of tests on acidified serum.--At the beginning of these experiments it was necessary to determine what effect, if any, slight acidification has on the neutralizing property of the serum. In each of the first five experiments a control test was made on this preparation. In one instance the serum failed to neutralize the virus; however, the results of other parts of the same experiment showed that the antibody had survived acidification. In this connection it may be mentioned that this same sample of serum, when used in other experiments as a control neutralizing serum, has failed to protect the test animal in one of

eight tests. Consequently, we do not feel that mere acidification has any appreciable effect on the neutralizing property of the serum. These control tests are represented in Column 8 of the Master Table.

TABLE V

ABSORPTION AND ELUTION OF THE ANTIBODY OF CONVALESCENT AND NORMAL HUMAN SERUMS WITH VIRUS-GEL AND ALUMINA-GEL.

		Convalescent human serum			Normal human serum			Summary		
			test animals			test animals			test animals	
Absorbed with	Fraction tested	number of experiments	number injected	number not infected	number of experiments	number injected	number not infected	number of experiments	number injected	number not infected
Virus-gel	Supernatant aH 6.5, plus test virus	6	13	8	5	11	9	9	24	17
	Eluate of sediment	5	9	8	3	8	7	6	17	15
Alumina-gel	Supernatant pH 6.5, plus test virus	7	13	3	5	10	4	11	23	7
	Eluate of sediment pH 7.4 plus test virus	5	12	10	5	11	8	9	23	18
Summary	Supernatants of V-G and A-G absorbed serum	10	26	11	6	21	13			
	Eluates of V-G and A-G sediments	9	21	18	6	19	15			

Discussion and Summary

The results of these experiments demonstrate conclusively that under proper conditions the antibody of convalescent and normal human serums may be absorbed and eluted with alumina-gel. The purity of the antibody in the eluate, however, has not been determined. Three observations relative to this should be mentioned here; that the gel eluate is invariably clearer than the absorbed serum, that the hemoglobin color of the serum is considerably diminished in the eluate, and that biuret tests are positive in much higher dilutions of the untreated serum than of the eluate. This indicates that the antibody is freed of at least some of the fats and proteins of the serum. Frankel and Olitzki claim that similar eluates of diphtheria antitoxin prepared with kaolin and glycine give no tests for protein.

The extent of antibody absorption from serum is not known, although it seems that as a rule most of it may be collected in the eluate of the adsorbate, as only three of thirteen animals receiving eluted antibody of convalescent serum and test virus and four of ten receiving the eluate of normal human serum and test virus, developed poliomyelitis, and on the other hand, about two-thirds of the tests of the absorbed serums were negative for antibody content. Similar experiments were done with normal monkey serum and no virucidal substance was found in either fraction.

The question arises, Can we collect all of the antibody which has been adsorbed to the gel? The one experiment in which the eluate was titrated indicated that this may be the case; however, the failure to reach an end point restricts the import to a certain extent.

No significant difference was observed in the behaviour

of the antibodies of convalescent and normal human serums toward the two absorbing agents --- virus-gel and alumina-gel. With serums which had been absorbed with alumina-gel, convalescent human serum gave only three instances of incomplete absorption in thirteen monkeys, and normal human serum gave only four in ten. Likewise, when the serums were absorbed with virus-gel the results were eight survivors among thirteen recipients of convalescent human serum and nine survivors among eleven receiving normal human serum. It is questionable whether or not these two types of serum preparations should be considered together, but if they are, the figures become eleven survivors among twenty-six animals receiving mixtures of test virus and convalescent serum which had been absorbed with either the virus-gel complex or alumina-gel, as compared with thirteen survivors of twenty-one monkeys treated with similar preparations of normal serum. The percentages are forty-two for the convalescent and sixty-two for the normal, which is not significant in this comparatively short series of tests. An analysis of the activity of the eluates of both adsorbates of the two serums gives an even smaller difference. Consequently, we may say that if the results obtained are at all applicable to the problem in question, they give evidence only of a similarity of the two types of antibodies under the conditions studied.

A real difference in result, however, does appear when the absorptions of serum with virus-gel are compared with the alumina-gel absorptions. Normal and convalescent serums were virus-neutralizing less than half as often when absorbed with alumina-gel as when absorbed with the virus-gel complex; of convalescent and normal human serum, respectively, only three of thirteen and four of ten animals survived when treated with alumina-gel ab-

sorbed serum, as compared with eight of thirteen and nine of eleven survivors among the groups treated with virus-gel absorbed serum. These figures become more significant if the two serums are considered as essentially the same in their reactivity to the virus and the absorbing agents. The total is seven survivors of twenty-three animals treated with alumina-gel absorbed serum as contrasted with seventeen survivors of twenty-three animals inoculated with serum which had been absorbed with the virus-gel complex. The percentages are thirty for the first group and seventy-five for the second. Thus it appears that the virus-gel complex has a weaker capacity for antibody absorption than either the normal-cord-gel complex or the alumina-gel. (This difference can hardly be ascribed to technical or biological variation because of the manner in which the experiments were controlled.)

Any consideration of the factors involved in these absorptions is more or less speculative, for the authorities on colloidal chemistry themselves are not agreed as to the influences which produce such phenomena. Nevertheless, it appears that an electrical force is the predominant factor (37); and this is particularly applicable to the present work because it is known that both the alumina-gel and the virus and most, if not all, antibodies do possess electrical charges.

The difference in capacity for antibody absorption between the virus-gel and the alumina-gel may be explained by assuming that the virus has satisfied a large portion of the charges of the gel, leaving an insufficient number available for union with the antibodies. On this basis it may be proposed that the difference in magnitude of charges is greater between the virus and the gel than it is between the antibody and the gel. This in turn implies a difference in the magnitudes of the virus charge

and the antibody charge, which furnishes a theoretical basis for a primary interaction between the two. The recovery under certain conditions of viable virus from neutral serum-virus mixtures by several investigators (8, 32, 38) lends support to this idea.

It is well recognized that the present evidence does not warrant any strong claims for the theory of charge phenomena as the force producing union between the virus and antibody of poliomyelitis, and it may be difficult to elicit additional evidence in support of this. If it could be shown that the virus-antibody complex is not adsorbable to alumina-gel, the theory would be strengthened considerably. Jaque and Zunz early showed that the diphtheria toxin-antitoxin complex was not adsorbable to charcoal, an agent that would adsorb either the toxin or the antitoxin alone. A few experiments were performed in the present work in an effort to develop a method of detecting the behaviour of the virus-antibody complex toward alumina-gel, but they were indeterminate.

GENERAL SUMMARY

Complement-fixation, precipitin, and skin tests have been attempted with poliomyelitis virus and antibody with negative results. Some of the virus preparations used in these tests had been concentrated and purified to some extent by ether treatment according to the method of Clifton, Schultz and Gebhardt, by the alumina-gel adsorption and elution method of Sabin, and by trypsinization and boiling *in vacuo*. It was found that the virus is capable of surviving at least eleven rapidly successive freezings and thawings.

In vivo protection tests were attempted by inoculating virucidal serum directly into the CNS tissue.

No instance of positive result was noted in any of these immunologic tests.

It has been found that the antibody of convalescent and normal human serums is adsorbable to alumina-gel and that it may be eluted from such a complex by making a simple change in the pH of the suspending fluid. Also, it has been found, comparatively, that the antibody is not as easily adsorbed by a virus-alumina-gel complex. These results have been discussed in regard to the specificity of the serum-neutralization test, and a theory has been advanced for the primary effect of union of the virus and antibody.

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MASTER TABLE*

| | Controls on Virus of Virus-gel Complex | | | | | | | | | Absorption and Elution of the Antibody of Convalescent Human Serum with | | | | | | | | |